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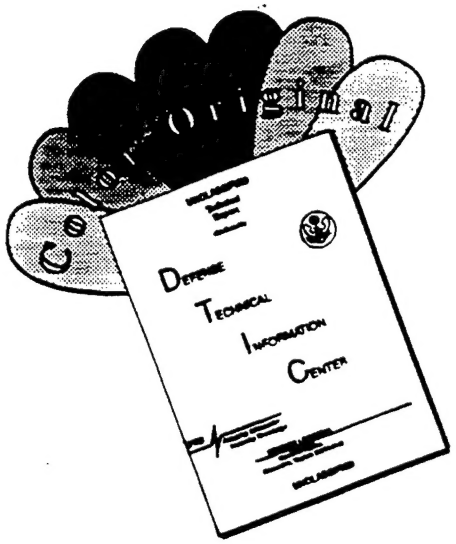

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This proposal is designed to investigate the role of PTHrP in breast cancer-mediated osteolysis. Observations in patients with bone metastases suggest that breast cancer cells in bone express PTHrP more frequently than in soft tissue sites of metastasis or in the primary tumor. Thus, the role of PTHrP in the causation of breast cancer metastases in bone was examined using human breast cancer cell lines. Four of eight breast cancer cell lines expressed PTHrP and one of these cell lines, MDA-MB-231, was studied *in vivo*. Mice inoculated with MDA-MB-231 cells developed osteolytic bone metastasis without hypercalcemia or increased plasma PTHrP concentrations. PTHrP concentrations in bone marrow plasma from femurs affected with osteolytic lesions were increased over corresponding plasma PTHrP concentrations. In a separate experiment, mice were treated with either a monoclonal antibody directed against PTHrP-(1-34), control IgG or nothing prior to tumor inoculation with MDA-MB-231 and twice per week for 26 days. Total area of osteolytic lesions was significantly lower in mice treated with PTHrP antibodies compared with mice receiving control IgG or no treatment. Histomorphometric analysis of bone revealed decreased osteoclast number per mm of tumor/bone interface, increased bone area as well as decreased tumor area in tumor-bearing animals treated with PTHrP antibodies compared with respective controls. These results indicate that PTHrP can cause local bone destruction in breast cancer metastatic to bone, even in the absence of hypercalcemia or increased circulating plasma concentrations of PTHrP.

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Theresa A Moore 10/28/96

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INTRODUCTION

NATURE OF THE PROBLEM AND BACKGROUND OF PREVIOUS WORK

Breast Cancer, Hypercalcemia and Osteolysis

Breast cancer is associated with significant morbidity in the skeleton. Specifically, breast cancer can involve bone through both metastatic and humoral mechanisms. Metastases to bone are more commonly osteolytic than osteoblastic and are responsible for the complications of bone pain, pathologic fracture, hypercalcemia and nerve compression syndromes that many breast cancer patients suffer from (1). Eighty-four per cent of patients dying of breast cancer have bone metastases (2).

Hypercalcemia is commonly associated with breast cancer, occurring in up to 40% of afflicted women during the course of their disease (2,3). Skeletal destruction by metastatic tumor has been felt to be the major mechanism responsible for hypercalcemia (3). Increased osteoclastic bone resorption in areas surrounding breast cancer metastasis has been documented histologically (4,5) suggesting that factors secreted by breast cancer cells can locally activate osteoclasts. Recent evidence, however, suggests that osteolytic bone metastasis may not be the only mechanism responsible for breast cancer hypercalcemia and that humoral mechanisms may contribute in as much as 30-60% of the cases (6-8). In one study, 15% of 147 hypercalcemic breast cancer patients had no bone metastases (9).

PTHrP and Breast Cancer

Parathyroid hormone-related protein (PTHrP) is a major mediator of humoral hypercalcemia of malignancy, due to its PTH-like actions. This protein was purified in 1987 from human lung cancer (10), breast cancer (11) and renal cell carcinoma (12) simultaneously by several independent groups. Cloning and expression followed shortly thereafter (13).

PTHrP has since been extensively studied and found to have many similarities to PTH. It has 70% homology to the first 13 amino acids of the N-terminal portion of PTH (13), binds to PTH receptors (14) and shares similar biologic activity to PTH (15). Specifically, it stimulates adenylate cyclase in renal and bone systems (11,12,15-17), increases renal tubular reabsorption of calcium and osteoclastic bone resorption (16,17), decreases renal phosphate uptake (15,16,18) and stimulates 1α-hydroxylase (15). PTHrP has been found in a variety of tumor types as well as normal tissue (19-22). The widespread expression of PTHrP in normal as well as malignant tissue was the first evidence that the hormone has a role in normal physiology. In addition to the PTH-like effects, PTHrP has many non-PTH-like properties (23), some of which include regulation of placental calcium transport (22), possible establishment of bone metastasis in breast cancer (24,25), and autocrine regulation of the growth of some tumors (26). The regulation of PTHrP is poorly understood, but factors such as prolactin (27), glucocorticoids, $1,25(OH)_2D_3$ (28), epidermal growth factor (28), TGFα (29), TGFβ (30), estrogen (31-34) and stretch (35) have been shown to regulate gene expression and extracellular calcium concentration has been shown to control the production of PTHrP in vitro in Leydig tumor cells (36).

It is now clear that PTHrP is a significant factor in mediating hypercalcemia in breast cancer (37). One of the 3 tumors from which PTHrP was originally purified was a breast cancer from a patient with humoral hypercalcemia of malignancy (11). PTHrP was detected by immunohistochemical staining in 60% of 102 invasive breast tumors removed from normocalcemic women, but not in normal breast tissue (24). By immunohistochemistry (25) and in situ hybridization (38), it was detected in 12 of 13 breast cancer metastases in bone prompting speculation that production of PTHrP as a bone-resorbing agent may contribute to the ability of breast cancers to grow as bone metastasis. Along these lines, Bundred and colleagues found positive immunohistochemical staining for PTHrP in 56% of 155 primary breast tumors from normocalcemic women and PTHrP positivity was related to the development of bone metastases (39). Additionally, 65-92% of hypercalcemic breast cancer patients (with and without bone metastasis) had detectable plasma PTHrP concentrations by radioimmunoassay (RIA) similar to those documented in patients with humoral hypercalcemia of malignancy due to non-breast tumors (40,41).

PTHrP in Nonmalignant Breast Disease

In addition to its role in malignancy, PTHrP is important in the normal physiology of breast (42). It is expressed in lactating mammary tissue (43) and secreted into milk at concentrations 10,000-100,000 times greater than plasma concentrations of humans with malignancy-associated hypercalcemia (44-48). Suckling increases PTHrP gene expression and this appears to be mediated through prolactin (49). Estrogen has been shown to increase PTHrP expression in uterine tissue and *in vitro* studies suggest that there may be estrogen response elements present in the PTHrP gene (50-53). Increased plasma PTHrP concentrations have been described in at least 2 patients with the rare syndrome of lactational hypercalcemia (54-56). Animal studies have demonstrated a PTHrP gradient across the mammary gland in lactating goats (48) indicating that PTHrP may gain access to the maternal circulation during lactation. In support of this, a recent clinical study has shown detectable plasma PTHrP concentrations in 63% of breast-feeding mothers while similar measurements in bottle-feeding control mothers were undetectable (57). Thus, PTHrP may be responsible for mobilizing calcium from maternal bone for use in milk production and it may be the implicating factor in lactation-associated bone loss (58).

PTHrP as a Growth Regulator

PTHrP is produced in relatively low concentrations in breast myoepithelial cells (59). A transgenic mouse model, in which PTHrP is over expressed in skin and breast myoepithelial cells through the use of a human keratin promoter, has demonstrated breast hypoplasia. Specifically, female transgenics had a severe reduction in the number of albeit normal terminal ducts and acini in the breast suggesting that PTHrP may play a role in regulating ductular proliferation and/or differentiation during mammogenesis (60). These mice also had failure of normal hair follicle development indicating a similar role for PTHrP in the skin.

Along those lines, disruption of PTHrP expression in a normal keratinocyte cell line, using antisense technology, results in enhanced growth of the cells in culture (61). *In vivo*, homozygous mice for the PTHrP null mutation are born with a multitude of skeletal abnormalities, including defects in the bone growth plate (62). These findings, along with those of the above described transgenic mice, suggest that either over- or under- expression of PTHrP in normal cells result in abnormalities of growth and possibly differentiation.

In malignant cells, PTHrP has been shown to act as an autocrine growth factor in a renal cell carcinoma cell line (26) and more recently, in a squamous cell carcinoma line (63). There are no reported studies on the role of PTHrP as an autocrine growth factor in breast cancer.

Regulation of PTHrP by Other Tumor-associated and Bone-derived Growth Factors

Other tumor-associated growth factors as well as bone-derived growth factors may be important regulators of PTHrP expression in both malignant and non-malignant tissue. Epidermal growth factor has been shown to increase PTHrP expression in a keratinocyte cell (64) line while TGF-α, a breast cancer tumor product (65), enhances PTHrP expression in a human squamous cell carcinoma of the lung (29). Moreover, other tumor-associated factors may modulate the end organ effects of PTHrP. TGF-α enhances the hypercalcemic effects of PTHrP in an animal model of malignancy-associated hypercalcemia (66) and it can modulate the renal and bone effects of PTHrP as well (67,68). Additionally, TGF-β, which is present in high concentrations in the bone microenvironment, has been shown to enhance secretion of and stabilize the message for PTHrP in a renal cell carcinoma (30) as well as in an epidermal squamous cell carcinoma (69).

Implications of PTHrP Status in Breast Cancer

These findings have important implications for the ability of breast cancer to affect the skeleton. First, breast cancers expressing PTHrP in addition to other tumor-associated factors, such as TGF-α (65), may be more likely to affect the skeleton through humoral and osteolytic mechanisms if the co-expressed factor enhances PTHrP expression in the primary tumor. Second, if estrogen regulates PTHrP expression in breast cancer cells as it does in other tissues, estrogen receptor positive tumors may preferentially express PTHrP. Finally, growth of breast cancer cells in bone may be enhanced if the tumor cells express PTHrP. TGF-β, as well as other bone derived growth factors, are present in high concentration in the bone microenvironment (70) and are released from bone during the process of osteoclastic bone resorption (71). PTHrP expression in breast cancer cells lodged in bone is likely to be increased in the presence of TGF-β. In this scenario, osteoclastic bone resorption is increased further causing release of more TGF-β and other growth factors into the bone microenvironment leading to further enhancement of PTHrP expression in the breast cancer cells. If PTHrP acts as an autocrine growth factor in breast cancer cells, as it does in some tumor models, then tumor growth would be enhanced as well. The clinical findings of an increased incidence of PTHrP expression in bone compared with other sites by Powell and colleagues (25,38) supports the notion that production of PTHrP as a bone resorbing agent may contribute to the ability of breast cancers to grow as bone metastases.

If PTHrP expression in the primary breast tumor indicates a propensity to metastasize to bone due to its potent bone resorbing capability, early treatment with inhibitors of bone resorption is likely to prevent or delay the development of bone metastases as well as reduce the catastrophic complications of pain, hypercalcemia, fracture and nerve compression syndromes. It is already clear from clinical studies that the use of bisphosphonates, potent inhibitors of bone resorption, significantly reduces skeletal morbidity in advanced breast cancer (72-74). Bisphosphonates have also been shown to decrease the number of bone metastases in animal models (75,76), but it is unclear whether or not these tumors express PTHrP. However, since the safety of long term bisphosphonate use has not been determined and bone mineralization defects can occur with high doses of these drugs, it would be of benefit, as well as cost effective, to identify which patients are at risk to develop bone metastases and treat only those rather than treat all women with breast cancer. The clinical evidence thus far supports PTHrP as a marker to identify such women, but better animal models are needed to

clarify this role.

Knowledge of PTHrP status may also have significant therapeutic implications in treating breast cancer-associated hypercalcemia. Although hypercalcemia in breast cancer is often associated with bone metastases, it is clear that humoral mechanisms may contribute in as much as 60% of the cases. Traditionally, treatment has been directed toward inhibiting bone resorption and this is often effective. However, it has now become evident that bisphosphonate therapy is less effective in patients with higher plasma concentrations of PTHrP and without radiological evidence of bone metastases (77,78). Thus, inhibition of bone resorption is effective when the major mechanism for hypercalcemia is increased bone resorption. Since PTHrP causes hypercalcemia by both increasing osteoclastic bone resorption and increasing renal tubular reabsorption of calcium, drugs that inhibit bone resorption alone may not normalize the calcium concentration if the plasma PTHrP concentration is high enough to add a significant renal component to the hypercalcemia. Drugs directed against either the actions of PTHrP or the secretion of PTHrP may therefore be more beneficial in the bisphosphonate resistant situation. Unfortunately, no such drugs are available at the current time but the need for them is obvious. A potentially useful therapy may prove to be the use of monoclonal antibodies against PTHrP. Sato has recently described successful use of an anti-PTHrP-(1-34) monoclonal murine antibody in an animal model of humoral hypercalcemia that ameliorated hypercalcemia and prolonged survival time in severely ill animals (79).

In Vivo Models of Hypercalcemia and Osteolysis

These observations demonstrate the need for further study of the role of PTHrP in malignant and nonmalignant breast disease. The only research done to date on PTHrP expression in human breast cancer and its potential role in humoral hypercalcemia and the development of osteolytic bone metastases have involved the small clinical studies described above (24,25,39-41). Despite numerous animal models of human breast cancer (80) that have been described to date, human breast cancer cell lines have not been studied *in vivo* for PTHrP expression and its relationship to the development of osteolytic bone metastases and humoral hypercalcemia. Most animal models of breast cancer have been used to evaluate the effect of various factors (81-83) on breast cancer growth. Only one spontaneous rat mammary tumor (Walker 256 carcinosarcoma) has been shown to cause humoral hypercalcemia in rats (84), produce PTHrP (85) and cause osteolytic bone metastases (75). Given the accumulating evidence documenting a humoral mechanism for hypercalcemia in breast cancer, the established role of PTHrP in humoral hypercalcemia of malignancy, the presence of PTHrP in malignant as well as lactating breast tissue and the presence of PTHrP in established breast cancer metastases to bone, it is evident that established models of human breast cancer should be evaluated for PTHrP expression and its relationship to the skeleton. Using animal models will be beneficial in defining this aspect of the pathophysiology of breast cancer and this will in turn have important prognostic and therapeutic implications.

Historically, it has been difficult to produce bone metastases in animal models of malignancy. Tumors inoculated subcutaneously or intramuscularly do not metastasize in nude mice and tumors inoculated into the tail vein usually produce only lung metastases. Yoneda has developed an animal model of human breast cancer cell metastasis to bone (76,86) which is based on a model originally described by Arguello (87). In this model, MDA-MB-231 breast cancer cells injected into the left ventricle of nude mice reliably produce osteolytic lesions that are evident radiologically as well as histologically. This model has been used to show that the bisphosphonate, risedronate, decreased osteolytic lesions when given simultaneously with tumor cells and inhibited both an increase in new bone metastases and progression of each metastatic focus when given to animals with pre-existing osteolytic lesions (76).

PURPOSE OF PRESENT WORK

Breast cancer affects the skeleton through humoral and local osteolytic mechanisms to cause the devastating complications of hypercalcemia, pain, fracture and nerve compression syndromes. PTHrP is an important humoral mediator of hypercalcemia in cancer and may have physiologic roles in the lactating breast as well as in cell growth and differentiation. The role of PTHrP in the pathophysiology of breast cancer is significant for several reasons. 1) PTHrP mediates hypercalcemia through its systemic effects of increasing osteoclastic bone resorption as well as renal tubular calcium reabsorption in at least 50% of hypercalcemic breast cancer patients even in the presence of bone metastases. 2) Due to its potent bone resorbing capacity, PTHrP expression in the primary tumor may aid in establishment of the bone metastases that are so characteristic of patients with breast cancer. 3) Growth factors present in the bone microenvironment further enhance PTHrP expression in breast cancer cells present in bone and promote development of osteolytic lesions and tumor growth. Thus, PTHrP expression in the primary breast tumor may be a marker for the development of hypercalcemia and bone metastases.

The purpose of this study is to define the role of PTHrP in the pathophysiology of breast cancer using animal models of breast cancer-mediated humoral hypercalcemia and osteolytic bone metastases. Information gained from these studies will have important prognostic and therapeutic implications.

METHODS OF APPROACH

In order to define the role of PTHrP in the pathophysiology of breast cancer-associated hypercalcemia and skeletal complications in a systematic fashion, the following objectives were originally proposed.

1. **SPECIFIC AIM #1: To screen known breast cancer cell lines for PTHrP expression and secretion and to determine if PTHrP expression is related to estrogen receptor status.**

   a. Known breast cancer cell lines (both estrogen receptor positive and negative) will be grown in culture along with positive and negative controls. Media conditioned for 24 hours will be screened for PTHrP immunoreactivity by immunoradiometric assay.

   b. RNA will be isolated from above cell lines in the presence and absence of estrogen and PTHrP expression will be determined using Northern analysis.

2. **SPECIFIC AIM #2: Determine if known human breast cancer cell lines will cause humoral hypercalcemia and if this is PTHrP-mediated.**

   a. Measure standard parameters of calcium homeostasis in nude mice bearing human breast tumors.

   b. Determine that hypercalcemia observed in mice bearing PTHrP+ breast tumors is PTHrP-mediated. Two approaches will be used: i) to decrease PTHrP secretion by transfecting PTHrP + lines with PTHrP antisense ii) decrease PTHrP effects by administration of neutralizing antibody.

      1. Transfection of PTHrP antisense cDNA into breast cancer cell lines that secrete PTHrP and cause hypercalcemia in nude mice.

      2. Measurement of Ca^{++} in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that have been transfected with PTHrP antisense cDNA.

      3. Measurement of Ca^{++} in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that are treated with anti-PTHrP-(1-34) monoclonal antibody.

3. **SPECIFIC AIM #3: To determine the role of PTHrP in the development of osteolytic metastases in breast cancer.**

a. Is PTHrP expression enhanced in the bone microenvironment relative to other metastatic sites? Using an animal model of breast cancer-mediated osteolysis, PTHrP expression will be compared in bone and non-bone sites using immunohistochemistry and in situ hybridization.

b. Does expression of PTHrP in the primary tumor enhance the development and quantity of osteolytic bone metastases? Breast cancer cell line, MDA-231 will be transfected with the cDNA for human PTHrP or PTHrP-AS (antisense orientation as a control) and used in the osteolytic model.

1. Production of stable MDA-231 clones expressing PTHrP or PTHrP-AS by calcium phosphate precipitation.

2. Effect of MDA-231/PTHrP on development of osteolytic bone metastases will be assessed by inoculating these cells into the left ventricle of mice and determining if the quantity and size of the bone metastases differ from similarly inoculated control MDA-231/PTHrP-AS. Neutralizing antibodies will be given to attempt to block osteolysis in mice inoculated with MDA-231/PTHrP cells.

BODY

METHODS

Cell culture

The following cell lines were cultured in the respective media: RWGT2 (66), MDA-MB-231 (88), CHO-K1 and Hs578T in DMEM (Life Technologies, Grand Island, NY); BT549, ZR-75-1, T-47D and BT483 in RPMI (JRH Biosciences, Lenexa, KS) with 10 µg/ml bovine insulin (Biofluids, Rockville, MD); MDA-MB-435s in Leibovitz L-15 (Life Technologies); MCF-7 in IMEM (Biofluids) with 10 µg/ml bovine insulin. All media contained 10% FCS (Hyclone, Logan, UT), 0.1% penicillin/streptomycin and nonessential amino acids (Gibco). Cells were cultured in a 37°C atmosphere of 5% CO_2/air. MDA-MB-231 cells were provided by C. K. Osborne, M.D. (San Antonio, TX) and all other cell lines except RWGT2 were obtained from the American Type Culture Collection (Rockville, MD). To determine PTHrP concentration in conditioned media, cell lines were simultaneously plated onto 48-well plates at cell density of 10^4/ml, grown to confluence and media (250 µl) were conditioned in the absence of serum for 48 hours. Cells were trypsinized and counted following collection of conditioned media. Media samples were stored at -70°C until assayed for PTHrP. For each cell line, PTHrP was measured in triplicate and corrected for cell number.

The effect of the bone-derived growth factor, TGFβ, on PTHrP secretion by breast cancer cell lines was investigated. Cells were plated in 48-well plates at 10^4 cells/ml and grown to confluence. Media was replaced with serum-free DMEM with or without TGFβ (5 ng/ml). Conditioned media were collected after 48 hours. PTHrP concentrations were determined by IRMA and corrected for cell number.

PTHrP measurement

Immunoreactive PTHrP was measured in conditioned media and plasma using a 2 site IRMA kit available from Nichols Institutes. This assay utilizes 2 different polyclonal antibodies to PTHrP (1-40 and 60-72) and will detect only peptides that bridge these antibodies. All samples, conditioned media, plasma and bone marrow plasma were collected in protease inhibitors and stored at -70°C until assay, as PTHrP is very labile. The assay is performed on aliquots of fifty to two hundred microliters. PTHrP concentrations were determined from a standard curve using a computerized RIA program. The sensitivity of the assay is 0.3 pmol/L and specificity to human PTHrP-(1-86) is 100% and 0% with PTH. Intra-assay variation is between 2.9-9.5% and inter-assay variation between 5.3-5.6%.

Whole blood ionized calcium measurement

Whole blood ionized calcium concentrations (Ca^{2+}) were measured using a Ciba Corning 634 ISE Ca^{++}/pH analyzer, which uses a sample volume of 35 μl. The calcium values are adjusted using the internal algorithm of the instrument pH of 7.4. Samples were run in duplicate and the mean value recorded. The analyzer were calibrated before and after running each group of samples using Ciba Corning 634 Slope Standard (Ca 2.50/pH 6.84). Many published studies have utilized this method (66,87,95).

Scoring of bone metastases

The number of osteolytic bone metastases was determined on radiographs as described by Nakai (86). At the end of the experiments, animals were anesthetized deeply with pentobarbital, laid down in a prone position against the films (22 x 27 cm X-O mart AR, Kodak) and exposed with an x-ray at 35 KVP for 6 seconds using a Faxitron Radiographic Inspection Unit (Model 8050-020, Field Emission Corporation, Inc.). Films were developed using a RP X-O Mart processor (Model M6B, Kodak). All of the radiographs of the bones in nude mice were evaluated extensively and carefully by 3 different individuals in a blinded fashion. From the radiographs, osteolytic metastatic foci as small as 1 mm in diameter, which are recognized as

demarcated radiolucent lesions in the bones, were enumerated.

Bone histology and histomorphometry. Forelimb and hindlimb long bones were removed from mice at sacrifice, fixed in 10% buffered formalin, decalcified in 14% EDTA and embedded in paraffin wax. Sections were cut using a standard microtome, placed on poly-L-lysine-coated glass slides and stained with hematoxylin, eosin, orange G and phloxine.

The following variables were measured in midsections of tibiae and femora, without knowledge of treatment groups, to assess tumor involvement: total bone area, total tumor area, and osteoclast number expressed per mm of tumor/bone interface. Histomorphometric analysis was performed on an OsteoMeasure System (Osteometrics, Atlanta, GA) using an IBM compatible computer.

In vivo experiments

A. Technical aspects of bone metastasis studies:

MDA-MB-231 breast cancer cells injected into the left ventricle of nude mice reliably produce osteolytic lesions that are evident radiologically as well as histologically. In this model, nude mice are inoculated into the left cardiac ventricle with a tumor cell suspension. In 3 weeks, osteolytic lesions are evident on radiographs. Histologic sections of these lesions reveal tumor adjacent to osteoclasts resorbing bone.

Tumor cells are grown to confluence, trypsinized, washed twice with PBS and resuspended with PBS to a final concentration of 10^5 cells/100µl immediately prior to inoculation. Animals are deeply anesthetized with pentobarbital (0.05 mg/g) and positioned ventral side up. The left cardiac ventricle is punctured through a percutaneous approach using a 27 gauge needle attached to a 1 ml syringe containing suspended tumor cells. Visualization of bright red blood entering the hub of the needle in a pulsatile fashion indicates correct position in the left cardiac ventricle. Tumor cells or PBS (for controls) are then inoculated slowly over 1 minute.

Three weeks following tumor inoculation, whole-body radiographs were obtained and compared to baseline radiographs obtained prior to tumor inoculation to follow the progression of osteolytic lesions. Ca^{++} and body weight were measured weekly for two weeks post tumor inoculation and then every three days thereafter for the remainder of the experiment. Animals were sacrificed when paraplegic or severely cachectic. At the time of sacrifice, blood was collected for Ca^{++} as well as PTHrP measurement and all bones and soft tissues were harvested in formalin for histologic analysis. Femurs with radiologic evidence of metastases from mice inoculated via the left cardiac ventricle with MDA-MB-231 cells or femurs from control mice were harvested by flushing marrow contents with 500 µl of serum-free DMEM media into iced tubes containing EDTA and aprotinin for measurement of PTHrP in bone marrow plasma. Autopsy was performed on all mice and those with tumor adjacent to the heart were excluded from analysis as this indicates that part of the initial tumor inoculum did not properly enter the left cardiac ventricle.

B. Experimental design:

1. Experiment 1: *PTHrP production in bone in vivo*. Mice were inoculated into the left cardiac ventricle on day 0 with either MDA-MB-231 cell suspension or PBS after baseline radiographs were obtained, and Ca^{2+} and plasma PTHrP concentrations were measured (n=5/group). Radiographs were taken at day 21 and at sacrifice to follow the progression of osteolytic lesions. Ca^{2+} and body weight were measured on days 7, 14, 21, 24, and at sacrifice, day 26. At sacrifice, blood was collected for Ca^{2+} and PTHrP measurement, bone marrow plasma was collected for PTHrP measurement and all bones and soft tissues were harvested in formalin for histologic analysis. Autopsies were performed on all mice, and those with tumor adjacent to the heart were excluded from analysis as this indicated that part or all of the initial tumor inoculum had not entered the left cardiac ventricle.

2. Experiment 2: ***Effects of PTHrP antibody on MDA-MB-231-induced bone metastases.***

a. On the development of new bone metastasis: Mice were divided into four treatment groups (n=7/group) and inoculated with MDA-MB-231 cells into the left cardiac ventricle on day 0. Treatment, administered at a dose of 75 µg subcutaneously twice per week starting 7 days prior to tumor inoculation and continued throughout the experiment, consisted of: 1) a murine monoclonal antibody directed against human PTHrP-(1-34) (PTHrP Ab; Mitsubishi, Japan); 2) control IgG (IgG; Sigma); 3) nothing; or 4) PTHrP Ab was given to a fourth group just prior to tumor inoculation and administered at the same dose and schedule for the remainder of the experiment. Radiography, Ca^{2+} and PTHrP measurement, bones and soft tissue harvest and autopsy were performed as in the previous experiment. Results from the 2 antibody groups were pooled in the final analysis as all parameters measured were similar regardless of whether the antibody treatment was initiated 7 days prior to or on the same day as tumor inoculation.

b. On the progression of established bone metastases: Eight female athymic nude mice were inoculated into the left cardiac ventricle on day 0 with nontransfected MDA-MB-231 cells. Osteolytic lesions were evident on radiographs at 2 weeks post inoculation and mice were divided into antibody treatment and control (PBS). Anti-PTHrP-(1-34) antibodies were administered subcutaneously twice per week beginning at this time in doses similar to those used in experiment 2a (70µg IgG).

Statistics

Unless otherwise indicated, values are reported as the mean ± standard error of the mean. Statistical significance was determined using analysis of variance followed by Tukey's post test with Graphpad Prism software (San Diego, CA) on an IBM compatible computer.

RESULTS

In vitro experiments

Production of PTHrP by human breast cancer cell lines in vitro: Of the 8 breast cancer cell lines tested for PTHrP secretion *in vitro*, 4 produced low, but significant, amounts of PTHrP (Table 1). The PTHrP concentration in media conditioned by MDA-MB-231 cells was 5.4±1.0 $pM/10^6$ cells/48 hours. This was significantly less than media conditioned by a squamous carcinoma of the lung, RWGT2, established from a patient with humoral hypercalcemia (66) (21.6±2.3 $pM/10^6$ cells/48 hr.) but more than secreted by Chinese hamster ovarian (CHO) cells (undetectable).

TABLE 1

CELL LINE	**[PTHrP] ($pM/10^6$ cells/48 hours)**
Human Breast Cancer	
MDA-MB-231	5.4 ±1.0**
Hs578T	4.6 ±0.5*
BT549	4.4 ±1.2*
MDA-MB-435s	2.9 ± 1.2
ZR-75-1	N.D.
BT483	N.D.
MCF-7	N.D.
T-47D	N.D.
Human Lung Cancer	
RWGT2	21.6 ±2.3***
Other	
CHOK1	N.D.

TABLE 1: PTHrP concentrations in conditioned media from cell lines. Collection of conditioned media is described in detail in the methods section. Results are expressed as the mean ± the standard error of the mean. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with CHOK1. N.D. = not detectable. N = 3 wells per cell line. PTHrP concentrations in conditioned media from each cell line were corrected for cell number.

Figure 1 illustrates PTHrP secretion by 9 different breast cancer cell lines in response to stimulation by TGFβ (5 ng/ml for 48 hours). All estrogen receptor positive cell lines (ER+), MDA-436, MDA-361, ZR-75-1, T-47D and BT-483, had undetectable concentrations of PTHrP in the conditioned media while all ER- lines, MDA-MB-231, BT-549, MDA-435s, Hs578T, and MDA-436 had low, but detectable PTHrP concentrations in the conditioned media. Of the 5 ER- cell lines that had measurable PTHrP, 4 of these cell lines increased production of PTHrP in response to TGFβ. TGFβ had no effect on PTHrP secretion by ER+ cell lines.

This work represents part of specific aim 1a that extends beyond studies reported in the last progress report. Estrogen regulation of PTHrP mRNA expression, as proposed in specific

aim 1b, is currently underway.

In vivo experiments

Experiment 1

MDA-MB-231 production of PTHrP in vivo: As part of specific aim 3a, PTHrP production by MDA-MB-231 cells was investigated *in vivo*. Mice inoculated with 10^5 MDA-MB-231 cells into the left cardiac ventricle developed radiographic evidence of osteolytic lesions over a period of 3 weeks. Mice were sacrificed at 4 weeks post tumor inoculation. Ca^{2+} and plasma PTHrP concentrations at sacrifice were not significantly different from respective values prior to tumor inoculation (1.28±0.05 mM vs. 1.29±0.03 mM for Ca^{2+}; 1.04±0.06 pM vs. 1.05±0.09 pM for PTHrP). In contrast, PTHrP concentrations in bone marrow plasma harvested from femurs affected with osteolytic lesions were significantly higher than corresponding plasma PTHrP concentrations (2.46±0.34 pM vs. 1.05±0.09 pM, $p<0.001$; Figure 2). Bone marrow plasma PTHrP concentrations from femurs of nontumor-bearing mice were below the detection limit of the assay. Thus, PTHrP produced by normal bone marrow cells was not of sufficient quantity to be detected by this method.

Experiment 2

PTHrP antibody experiments in vivo: As part of specific aim 3b, to determine if expression of PTHrP in the primary breast tumor enhances the development and quantity of osteolytic bone metastases, mice inoculated with MDA-MB-231 cells were studied in the mouse metastasis model with or without treatment with PTHrP antibodies. Since experiment 1 suggested that local PTHrP production by cancer cells in bone may be important in breast cancer-mediated osteolysis, the next experiment was designed to determine the role of PTHrP in the development of MDA-MB-231-mediated osteolysis. Nude mice were treated with a murine monoclonal antibody directed against PTHrP-(1-34) prior to intracardiac inoculation of tumor cells and compared with similarly inoculated animals treated with control IgG or nothing. PTHrP Ab and control IgG were administered at a dose of 75 µg twice per week throughout the experiment. Mice were sacrificed 26 days after tumor inoculation. One mouse each from the no treatment group and the PTHrP Ab group were excluded from analysis as tumor was adjacent to the heart at autopsy.

Figure 3 illustrates representative radiographs taken 26 days after tumor inoculation. Obvious osteolytic lesions were present in mice that received no treatment or control IgG, while very few metastatic lesions were present in mice treated with the PTHrP Ab. The total area of radiographic osteolytic lesions from all long bones was quantified by a computerized image analysis system (Figure 4). Lesion area was significantly less in mice treated with the PTHrP Ab compared with mice given no treatment or control IgG ($p<0.001$). Values in the latter two groups were not statistically different. Representative histologic sections through the proximal tibial metaphysis are illustrated in Figure 5. Tumor filled the bone marrow space and destroyed both trabecular and cortical bone in mice that received no treatment or control IgG. In contrast, most of the PTHrP Ab-treated mice had intact cortical and trabecular bone and many bones had no evidence of tumor involvement. When tumor was present in the bone marrow space in PTHrP Ab-treated mice, it was often present as small, discrete foci within the marrow cavity and associated with little or no bone destruction.

Histomorphometric analysis of the hindlimbs from mice in all treatment groups confirmed radiographic quantitation of the osteolytic lesion area (Figure 6). Tumor area (Figure 6A) and osteoclast number per mm of tumor/bone interface (Figure 6B) were significantly less in mice treated with PTHrP Ab compared with the mice that received no treatment or control IgG. Residual bone area was significantly higher in the PTHrP Ab-treated mice compared with the controls (Figure 6C).

Ca^{2+} concentrations remained normal in all groups for the duration of the experiment (1.26±0.03mM (PTHrP Ab), 1.28±0.02 mM (no treatment), 1.28±0.02 mM (IgG); p=ns; Figure 7A). Body weight significantly declined in mice that received no treatment or control IgG

compared with those treated with PTHrP Ab (Figure 7B). These weight differences reached statistical significance on day 26 (21.6±0.6 g (PTHrP Ab) vs. 18.8±0.8g (no treatment) and 17.7±0.8g (IgG); $p<0.05$). Additionally, no differences were evident between PTHrP Ab-treated and control mice regarding tumor cell metastases to sites other than bone. Gross and histologic examination of soft tissues revealed adrenal gland metastasis in one mouse each from the no treatment group and the PTHrP Ab group.

In a separate experiment to determine if treatment with PTHrP antibodies could delay the progression of established MDA-MB-231 bone metastasis, female athymic nude mice with radiographic osteolytic lesions 2 weeks post tumor inoculation were treated with either PTHrP-Ab or control (PBS) subcutaneously twice per week. Figure 8 demonstrates that total radiographic lesion area was less in those animals treated with the PTHrP-Ab compared with controls ($p<0.001$).

CONCLUSIONS

These findings demonstrate that nude mice, following inoculation with MDA-MB-231 cells into the left ventricle, develop metastatic bone disease with the same characteristic features as those seen in breast cancer patients. Tumor-bearing mice not only have osteolysis without increased plasma calcium or PTHrP concentrations but also have enhanced production of PTHrP by tumor cells in the bone microenvironment. In our experiments, this was evidenced by the increased PTHrP concentrations in the bone marrow plasma of affected femurs compared with blood plasma. Thus, the concentrations of PTHrP secreted *in vivo* by MDA-MB-231 cells are presumably sufficient to mediate local osteolysis, but not enough to have the systemic effects that characterize humoral hypercalcemia. Treatment with PTHrP monoclonal antibodies in this model of breast cancer-mediated osteolysis resulted in marked inhibition of the development of new osteolytic lesions and decreased osteoclast number per millimeter of tumor/bone interface, indicating that PTHrP is the critical mediator of bone destruction in this situation.

Several clinical studies in breast cancer patients indicate that plasma PTHrP concentrations are increased in approximately 50% of those with hypercalcemia (89-91). These data, along with the fact that one of the 3 tumors from which PTHrP was originally purified (10-12) was a breast carcinoma associated with humoral hypercalcemia, show that PTHrP may mediate hypercalcemia in some patients with breast cancer. In addition to this established role of PTHrP in malignancy-associated hypercalcemia, the findings presented here implicate PTHrP in the causation of breast cancer-mediated osteolysis even in the absence of hypercalcemia or increased plasma PTHrP concentrations.

One issue which arises is whether local PTHrP production by breast cancer cells in bone is a common phenomenon, and how many patients with metastatic breast cancer to bone would benefit from neutralization or inhibition of PTHrP. This issue remains to be resolved. However, our survey of PTHrP production in breast cancer cell lines demonstrate that 5 out of 9 cell lines tested secrete significant amounts of PTHrP. These data support the clinical studies which demonstrate PTHrP expression in primary breast cancer by immunohistochemical methods to be approximately 50-60% (24). The clinical observations that primary breast tumors which express PTHrP are associated with the development of bone metastases (39,40) and that PTHrP expression by breast cancer cells in bone is greater than that of tumor cells which have metastasized to non-bone sites (25) or primary breast tumors (24) are consistent with the *in vivo* data presented here.

These data and those of others show that PTHrP is frequently produced by human breast cancer cells *in vitro* and *in vivo*, and that neutralization of PTHrP may inhibit development or progression of osteolytic metastases, but they do not exclude the involvement of other mediators. Cytokines, such as tumor necrosis factor, interleukin-6 or interleukin-1 produced locally by tumor cells or normal host cells, in response to the tumor, have the capacity to stimulate osteoclastic bone resorption (92). Such mediators have also been shown to

modulate the end-organ effects of PTHrP (66,93) as well as to increase its secretion from tumor cells (94). Thus, other locally-produced osteolytic factors may contribute to breast cancer-induced bone destruction as well.

Immunohistochemical data from patients with metastatic breast cancer suggest that PTHrP production by tumor cells is enhanced in the bone microenvironment (25). Our data are in agreement with this concept. The capacity of breast cancer cells to express PTHrP may give them a growth advantage after they have metastasized to bone, due to the ability of PTHrP to increase osteoclastic bone resorption (17). Growth factors, such as transforming growth factor beta (TGFβ) and insulin-like growth factors I and II are present within bone matrix (70) and released into the bone microenvironment as a result of osteoclastic bone resorption (71). Such bone-derived growth factors are likely to be in high concentration and in close proximity to tumor cells in bone. TGFβ enhances PTHrP expression by breast (95) and other cancers (96) and insulin-like growth factors may modulate breast cancer growth (97). Thus, once cancer cells in bone stimulate osteoclastic bone resorption, they may initiate a vicious cycle in which growth factors released from matrix enhance tumor cell growth and PTHrP production. This leads to more aggressive local bone resorption and a more favorable environment for further tumor growth and subsequent bone destruction. Since normal bone is actively remodeling, and growth factors are being released locally, PTHrP expression may be stimulated once the breast cancer cells lodge in the bone marrow stroma.

We found a marked decrease in tumor area in mice treated with PTHrP Ab, which is consistent with the notion that tumor growth is positively correlated with rates of bone resorption. In further support that neutralization of PTHrP leads to a decrease in tumor burden, tumor-bearing mice treated with PTHrP Ab maintained normal weight whereas the controls lost a significant amount of body weight. A possible explanation is that the neutralizing antibodies to PTHrP had a direct effect on tumor growth, but this is unlikely since tumors growing in non-bone sites were not affected by PTHrP Ab, and PTHrP Ab did not affect tumor cell proliferation *in vitro*. The most likely explanation is that the antibodies neutralized the biological activity of PTHrP, thereby preventing the increase in osteoclastic bone resorption and the release of growth factors from bone which may enhance growth of the tumor cells locally. This mechanism is supported by studies demonstrating that tumor burden in bone was decreased in mice treated with bisphosphonates, selective inhibitors of osteoclastic bone resorption (76).

The observation that normal body weight was maintained in tumor-bearing mice suggest that limiting development of bone metastases with PTHrP Ab did not lead to enhanced tumor growth in other organ sites. However, the overall effect of this treatment on tumor metastasis to organ sites other than bone remains to be explored. What is clear is that neutralizing antibodies prevented destructive bone lesions and also reduced tumor mass in bone.

These data have important implications for the management of patients with breast cancer-mediated osteolysis. First, treatment directed against PTHrP, such as the antibodies used in these experiments, may prevent the development of new bone metastases and delay the progression of established metastases. Second, PTHrP expression by the primary breast tumor may be a marker for increased capacity to form bone metastases. Third, treatment with inhibitors of PTHrP or inhibitors of osteoclastic bone resorption such as bisphosphonates may be effective adjuvant therapies not only for the prevention and treatment of bone metastases, but also for reducing tumor burden by making bone a less favorable site for continued tumor growth.

REFERENCES

1. Guise TA and Mundy GR. Breast cancer and bone. Current Opinion in Endocrinology and Metabolism. 2:548-555, 1995.

2. Aaron AD. The management of cancer metastatic to bone. JAMA 272(15):1206-1209, 1994.

3. Yoneda T, Sasaki A, Mundy GR. Osteolytic metastases in breast cancer. Breast Cancer Res Treat 32(1):73-84, 1994.

4. Galasko CSB. Mechanisms of bone destruction in the development of skeletal metastasis. Nature 263:507-508 1976

5. Taube T, Elomaa I, Blomqvist C, Beneton MNC, Kanis JA. Histomorphometric evidence for osteoclast-mediated bone resorption in metastatic breast cancer. Bone 15(2):161-166, 1994.

6. Isales C, Carcangiu ML, Stewart AF. Hypercalcemia in breast cancer: reassessment of the mechanism. Am J Med 82:1143-1146 1987

7. Kimura S, Adchi I, Yamaguchi K, Suzuki M, Shimada A, Sato Y, Nagaoka K, Abe K. Stimulation of calcium reabsorption observed in advanced breast cancer patients with hypercalcemia and multiple bone metastasis. Jpn J Cancer Res 76:308-314 1985

8. Gallacher SJ, Fraser WD, Patel U, Logue FC, Soukop M, Boyle IT, Ralston SH. Breast cancer-associated hypercalcemia: a reassessment of renal calcium and phosphate handling. Annals of Clinical Biochemistry 27:551-6 1990

9. Coleman RE, Fogelman I, Rubens RD. Hypercalcemia and breast cancer - an increased humoral component in patients with liver metastasis. Eur J Surg Oncol 14:423-428 1988

10. Moseley JM, Kubota M, Diefenbach-Jagger H, Wettenhall REH, Kemp BE, Suva LJ, Rodda CP, Ebeling PR, Hudson PJ, Zajac JD, Martin TJ. Parathyroid hormone-related protein purified from a human lung cancer cell line. Proc Natl Acad Sci USA 84:5048-5052, 1987

11. Burtis WJ, Wu T, Bunch C, Wysolmerski J, Insogna K, Weir E, Broadus AE, Stewart AF. Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. J Biol Chem 262(15):7151-7156 1987

12. Strewler GJ, Stern P, Jacobs J, Eveloff J, Klein RF, Leung SC, Rosenblatt M, Nissenson R. Parathyroid hormone-like protein from human renal carcinoma cells structural and functional homology with parathyroid hormone. J Clin Invest 80:1803-1807, 1987

13. Suva LJ, Winslow GA, Wettenhall REH, Hammonds RG, Moseley JM, Dieffenbach-Jagger, Rodda C, Kemp BE, Rodriguez H, Chen E, Hudson PJ, Martin TJ, Wood WI. A parathyroid hormone-related protein implicated in malignant hypercalcemia: Cloning and expression. Science 237:893-896, 1987

14. Abou-Samra A, Juppner H, Force T, Freeman MW, Kong X, Schipani E, Urena P, Richards J, Bonventre JV, Potts JT, Kronenberg HM, Segre GV. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: A single receptor stimulates intracellular accumulation of both cAMP and inositol triphosphates and increases intracellular free calcium. Proc Natl Acad Sci USA 89:2732-2736, 1992

15. Horiuchi N, Caulfield M, Fisher JE, Goldman M, Mckee R, Reagan J, Levy J, Nutt R, Rodan S, Schoefield T, Clemens T, Rosenblatt M. Similarity of synthetic peptide from human tumor to parathyroid hormone in vivo and in vitro. Science 238:1566-1568, 1987

16. Kemp BE, Moseley JM, Rodda CP, Ebeling PR, Wettenhall REH, Stapleton D, Dieffenbach-Jagger H, Ure F, Michelangeli BP, Simmons HA, Raisz LG, Martin TJ. Parathyroid hormone-related protein of malignancy: Active synthetic fragments. Science 238:1568-1570, 1987

17. Yates AJP, Gutierrez GE, Smolens P, Travis PS, Katz MS, Auftemorte TB, Boyce BF, Hymer TK, Poser JW, Mundy GR. Effects of a synthetic peptide of a parathyroid hormone-related protein on calcium homeostasis, renal tubular calcium reabsorption, and bone metabolism in vivo and in vitro in rodents. J Clin Invest 81:932-938, 1988

18. Sartori L, Weir EC, Stewart AF, Broadus AE, Mangin M, Barrett PQ, Insogna KL. Synthetic and partially-purified adenylate cyclase-stimulating proteins from tumors associated with humoral hypercalcemia of malignancy inhibit phosphate transport in a PTH-responsive renal cell line. J Clin Endocrinol Metab 66:459-461, 1987

19. Asa SL, Henderson J, Goltzman D, Drucker DJ. Parathyroid hormone-like peptide in normal and neoplastic human endocrine tissues. J Clin Endocrinol Metab 71:1112-1118, 1990

20. Thiede MA and Rodan GA. Expression of a calcium mobilizing parathyroid-like peptide in lactating mammary tissue. Science 242:161, 1988

21. Danks JA, Ebeling PR, Hayman J, Chou ST, Moseley JM, Dunlop J, Kemp BE, Martin TJ. Parathyroid hormone-related protein: Immunohistochemical localization in cancers and in normal skin. J Bone Min Res 4:273-278, 1989

22. Rodda CP, Kubota M, Heath JA, Ebeling PR, Moseley JM, Care AD, Caple IW, Martin TJ. Evidence for a novel parathyroid hormone-related protein in fetal lamb parathyroid glands and sheep placenta: comparisons with a similar protein in humoral hypercalcemia of malignancy. J Endocrinol 117:261-71, 1988

23. Mallette LE. The parathyroid polyhormones: New concepts in the spectrum of peptide hormone action. Endo Rev 12:110-117, 1991

24. Southby J, Kissin MW, Danks JA, Hayman JA, Mosely JM, Henderson MA, Bennett RC, Martin TJ. Immunohistochemical localization of parathyroid hormone-related protein in breast cancer. Can Res 50:7710-7716 1990

25. Powell GJ, Southby J, Danks JA, Stillwell RG, Hayman JA, Henderson MA, Bennett RC, Martin TJ. Localization of parathyroid hormone-related protein in breast cancer metastasis: increased incidence in bone compared with other sites. Can Res 51:3059-3061 1991

26. Burton PBJ, Moniz C, Klight D. Parathyroid hormone-related peptide can function as an autocrine growth factor in human renal cell carcinoma. Biochem Biophys Res Commun 167:1134-1138, 1990

27. Theide MA. The mRNA encoding a parathyroid hormone-like peptide is produced in mammary tissue in response to elevations in serum prolactin. Mol Endocrin 3:1443-1447, 1989

28. Kremer R, Karaplis AC, Henderson J, Gulliver W, Banville D, Hendy GN, Goltzman D. Regulation of parathyroid hormone-like peptide in cultured normal human keratinocytes. J Clin Invest 87:884-893 1991

29. Burton PBJ, Knight DE. Parathyroid hormone-related peptide can regulate growth of human lung cancer cells. FEBS 305:228-232, 1992

30. Zakalik D, Diep D, Hooks MA, Nissenson RA, Strewler GJ. Transforming growth factor β increases stability of parathyroid hormone-related protein messenger RNA. J Bone Min Res 7 (Suppl 1):104A, S118, 1992

31. Fukayama S, Tashjian AH. Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone (PTH) and PTH-related protein. Endocrinology 124:397-401, 1989

32. Thiede MA, Harm SC, Hasson DM, Gardner RM. in vivo regulation of parathyroid hormone-related peptide messenger ribonucleic acid in the rat uterus by 17β-estradiol. Endocrinology 128:2317-2323, 1991

33. Weir E, Daifotos A, Dreyer B, Burtis W, Broadus A. Estrogen-responsive expression of the parathyroid hormone-related peptide gene by cultured primary myometrial cells. J Bone Min Res 6 (Suppl 1):598A, 1991

34. Thiede MA, Harm SC, Gardner RM. In vivo regulation of the parathyroid hormone-related protein by estrogens and antiestrogens. J Bone Min Res 6 (Suppl 1):599A, 1991

35. Daifotis AG, Weir EC, Dreyer BE, Broadus AE. Stretch-induced parathyroid hormone-related protein gene expression in the rat uterus. J Biol Chem (33):23455-8 1992

36. Rizzoli R and Bonjour JP. High extracellular calcium increases the production of a parathyroid hormone-like activity by cultured Leydig tumor cells associated with humoral hypercalcemia. J Bone Min Res 4:839-844, 1989

37. Heath DA. Parathyroid hormone related protein. Clinical Endocrinology 38:135-6 1993

38. Vargas SJ, Gillespie MT, Powell GJ, Southby J, Danks JA, Moseley JM, Martin TJ. Localization of parathyroid hormone-related protein mRNA expression and metastatic lesions by in situ hybridization. J Bone Min Res 7(8):971-980 1992

39. Bundred NJ, Walker RA, Ratcliffe WA, Warwick J, Morrison JM, Ratcliffe JG. Parathyroid hormone related protein and skeletal morbidity in breast cancer. European Journal of Cancer 28(2-3):690-92 1992

40. Bundred NJ, Ratcliffe WA, Walker RA, Cjoley S, Morrison JM, JRatcliffe JG. Parathyroid hormone related protein and hypercalcaemia in breast cancer. British Medical Journal 303(6816):1506-9 1991

41. Grill V, Ho P, Body JJ, Johanson N, Lee SC, Kukreja SC, Mosely JM, Martin TJ. Parathyroid hormone-related protein; elevated levels in both humoral hypercalcemia and hypercalcemia complicating metastatic breast cancer. J Clin Endo Met 73:1309-1315 1991

42. Ratcliffe WA. Role of parathyroid hormone-related protein in lactation. Clinical Endocrinology 37:402-404 1992

43. Thiede MA, Rodan GA. Expression of a calcium-mobilizing parathyroid hormone-like peptide in lactating mammary tissue. Science 242:278-280 1988

44. Budayr AA, Halloran BP, King JC, Diep D, Nissenson RA, Strewler GJ. High levels of parathyroid hormone-like protein in milk. Proc Natl Acad Sci USA 86:7183-7185 1989

45. Yamamoto M, Fisher JE, Thiede MA, Caulfield MP, Rosenblatt M, Duong LT. Concentrations of parathyroid hormone-related protein in rat milk change with duration of lactation and interval from previous suckling, but not with milk calcium. Endocrinol 130:741-747 1992

46. Khosla S, Johansen KL, Ory SJ, Obrien PC, Kao PC. Parathyroid hormone-related peptide in lactation and in umbilical cord blood. Mayo Clin Proc 65:1408-1418 1990

47. Stewart AF, Wu TL, Insogna KL, Milstone LM, Burtis WJ. Immunoaffinity purification of parathyroid hormone-related protein from bovine milk and human keratinocyte-conditioned medium. J Bone Min Res 6:305-311 1991

48. Ratcliffe WA, Thompson GE, Care AD, Peaker M. Production of parathyroid hormone-related protein by the mammary gland of the goat. Journal of Endocrinology 133:87-93 1992

49. Thiede MA. The mRNA encoding a parathyroid hormone-like peptide is produced in mammary tissue in response to elevations in serum prolactin. Mol Endo 3:1443-47 1989

50. Fukayama S, Tashjian AH. Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone(PTH) and PTH-related protein. Endocrinol 124:397-401 1989

51. Thiede MA, Harm SC, Hasson DM, Gardner RM. In vivo regulation of parathyroid hormone-related peptide messenger ribonucleic acid in the rat uterus by 17β-estradiol. Endocrinol 128:2317-2323 1991

52. Weir E, Daifotos A, Dreyer B, Burtis W, Broadus A. Estrogen-responsive expression of the parathyroid hormone-related peptide gene by cultured primary myometrial cells. J Bone Min Res 6:suppl 1 A598 1991

53. Thiede MA, Harm SC, Gardner RM. In vivo regulation of the parathyroid hormone-related protein by estrogens and antiestrogens. J Bone Min Res 6:suppl 1 A599 1991

54. Lepre F, Grill V, Danks JA. Hypercalcemia in pregnancy and lactation due to parathyroid hormone-related protein production. Bone Min 10:s317 1990

55. Khosla S, van Heerden JA, Garib H, Jackson IT, Danks J, Hayman JA, Martin TJ. Parathyroid hormone-related protein and hypercalcemia secondary to massive mammary hyperplasia. N Engl J Med 322(16):1157 1990

56. Lepre F, Grill V, Ho PWM, Martin TJ. Hypercalcemia in pregnancy and lactation associated with parathyroid hormone-related protein. N Engl J Med 328(9):666-667 1993

57. Grill V, Hillary J, Ho PMW, Law FMK, MacIsaac RJ, MacIsaac IA, Moseley JM, Martin TJ. Parathyroid hormone-related protein: a possible endocrine function in lactation. Clinical Endocrinology 37:405-410 1992

58. Strewler GJ and Nissenson RA. The parathyroid hormone-related protein as a regulator of normal tissue functions. Current Opinion in Endocrinology and Diabetes 286-292 1993

59. Seitz PK, Cooper KM, Ives KL, Ishizuka J, Townsend CM, Rajaraman S, Cooper CW. Parathyroid hormone-related peptide production and action in a myoepithelial cell line derived from normal human breast. Endocrinology 133:1116-1124 1993

60. Wysolmerski J, Daifotis A, Broadus, Milstone L, Philbrick W. Overexpression of PTHrP in transgenic mice results in breast hypoplasia. J Bone Min Res 8(Supplement 1):129A 1993

61. Kaiser SM, Laneuville P, Bernier SM, Rhim JS, Kremer K, Goltzman D. Enhanced growth of a human keratinocyte cell line induced by antisense RNA for parathyroid hormone-related peptide. J Biol Chem 267:13623-13628, 1992

62. Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL Kronenberg HM, Mulligan RC. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev 8:277-89 1994.

63. Moy AA, Kats Y, Vasavada R, Dann P, Burtis WJ, Philbrick WM, Orloff JJ. Parathyroid hormone-related protein (PTHrP) is a potent autocrine growth promoter in a squamous carcinoma cell line. J Bone Min Res 8(Supplement 1):235A 1993

64. Allinson ET, Drucker DJ. Parathyroid hormone-like peptide shares features with members of early response gene family: Rapid induction by serum growth factors and cyclohexamide. Can Res 52(11):3103-9 1992

65. Nagata N, Yasutumo Y, Kugai N, Matsura Y, Akatsu T, Su G, Yama K, Kinoshita T, Takatami O, Mamiya Y, Yamaguchi K. Parathyroid hormone-related protein and transforming growth factor activities in an extract from a breast cancer associated with humoral hypercalcemia of malignancy. Jpn J Clin Oncol 19:353-359, 1989

66. Guise TA, Yoneda T, Yates AJP, Mundy GR. The combined effect of tumor produced parathyroid hormone-related protein and transforming gorwth factor α enhance hypercalcemia in vivo and bone resorption in vitro. J Clin Endocrinol Metab 77:40-45 1993

67. Pizurki L, Rizzoli R, Caverzasio J, Bonjour J. Effect of transforming growth factor α and parathyroid hormone-related protein on phosphate transport in renal cells. Am J Physiol 259:F929-F935, 1990

68. Pizurki L, Rizzoli R, Caverzasio J, Bonjour J. Stimulation by parathyroid hormone-related protein and transforming growth factor α of phosphate transport in osteoblast-like cells. J Bone Min Res 6:1235-1241, 1991

69. Kiriyama T, Gillespie MT, Glatz, JA, Fukumoto S, Moseley JM, Martin TJ. Transforming growth factor β stimulation of parathyroid hormone-related protein (PTHrP): a paracrine regulator? Molecular and Cellular Endocrinology 92:55-62 1992

70. Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, Klagsbrun M. Growth factors in bone matrix. J Biol Chem 261:12665-12674 1986

71. Pfeilschifter J, Mundy GR. Modulation of transforming growth factor β activity in bone cultures by osteotropic hormones. Proc Natl Acad Sci 84:2024-2028 1987

72. van Holten-Verzantvoort AT, Bijvoet OLM, Hermans J, Harinck HIJ, Elte JWF, Beex LVAM, Cleton FJ, Kroon HM, Vermey P, Neijt JP, Blijham G. Reduced morbidity from skeletal metastases in breast cancer patients during long-term bisphophonate (APD) treatment. Lancet 8566:983-985 1987

73. Cleton FJ, van Holten-Verzantvoort AT, Bijvoet OLM. Effect of long-term bisphosphonate treatment on morbidity due to bone metastases in breast cancer patients. Recent Results in Cancer Research 116:73-78 1989

74. Burckhardt P, Thiebaud D, Perey L, von Fliedner V. Treatment of tumor-induced osteolysis by APD. Recent Results in Cancer Research 116:54-66 1989

75. Bassani D, Sabatini M, Scanziani E, De Francesco L, Coccioli G, Guaitani A, Bartosek I. Bone invasion by Walker 256 carcinoma, line A in young and adult rats: effects of etidronate. Oncology 47:160-165 1990

76. Sasaki A, Boyce BF, Story B, Wright KR, Chapman M, Boyce R, Mundy GR, Yoneda T. The bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Can Res (in press) 1995

77. Gurney H, Grill V, Martin TJ. Parathyroid hormone-related protein and response to pamidronate in tumour-induced hypercalcaemia. Lancet 341:1611-1613 1993

78. Dodwell DJ, Abbas SK, Morton AR, Howell A. Parathyroid hormone-related protein$^{(50-69)}$ and response to pamidronate therapy for tumour-induced hypercalcaemia. Eur J Cancer 27(12):1629-1633 1991

79. Sato K, Yamakawa Y, Shizume K, Satoh T, Nohtomi K, Demura H, Akatsu T, Nagata N, Kasahara T, Ohkawa H, Ohsumi K. Passive immunization with anti-parathyroid hormone-related protein monoclonal antibody markedly prolongs survival time of hypercalcemic nude mice bearing transplanted human PTHrP-producing tumors. J Bon Min Res 8(7):849-860 1993

80. Engel LW, Young NA. Human breast carcinoma cells in continuous culture: a review. Can Res 38:4327-4339 1978

81. Furlanetto RW, DiCarlo JN. Somatomedin-C receptors and growth effects in human breast cells maintained in long term tissue culture. Can Res 44:2122-2128 1984

82. Lippman ME, Dickson RB, Gelmann EP, Rosen N, Knabbe C, Bates S, Bronzert D, Huff K, Kasid A. Growth regulation of human breast carcinoma occurs through regulated growth factor secretion. J Cell Biochem 35:1-16 1987

83. Paciotti GF and Tamarkin L. Interleukin-1 directly regulates hormone-dependent human breast cancer cell proliferation in vitro. Mol Endo 2:459-464 1988

84. Minne H, Rane F, Bellwinkel S, Ziegler R. The hypercalcemic syndrome in rats bearing the Walker carcinosarcoma 256. Acta Endocrinol 78:613-619 1975

85. DeMiguel F, Esbrit P. Purification of parathyroid hormone-related protein from the hypercalcemic rat strain of the rat Walker 256 carcinosarcoma. J Bone Min Res 6:suppl 1 A601 1991

86. Nakai M, Mundy GR, Williams PJ, Boyce B, Yoneda T. A synthetic antagonist to laminin inhibits the formation of osteolytic metastases by human melanoma cells in nude mice. Cancer Research 52:5395-5399 1992

87. Arguello F, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. Cancer Research 48:6878-6881 1988

88. Cailleau R, Yong R, Olive M, Reeves WJ. Breast tumor cell lines from pleural effusions. J Natl Cancer Inst 53:661-674, 1974

89. Stewart, A.F. Horst, R., Deftos, L.J., Cadman, E.C., Lang, R., and A.E. Broadus. Biochemical evaluation of patients with cancer-associated hypercalcemia: evidence for humoral and nonhumoral groups. N. Engl. J. Med. 303:1377-1383,1980.

90. Pandian, M.R., Morgan, C.H., Carlton, E., and G.V.Segre.Modified immunoradiometric assay of parathyroid hormone-related protein: clinical application in the differential diagnosis of hypercalcemia. Clin. Chem. 38:282-288, 1992.

91. Budayr, A.A., Nissenson, R.A., Klein, R.F. Increased serum levels of a parathyroid hormone-like protein in malignancy-associated hypercalcemia. Ann. Int. Med. 111:807-812, 1989

92. Manolagas, S.C. Role of cytokines in bone resorption. Bone 17:63S-67S,1995.

93. DeLaMata, J., H.L. Uy, T.A. Guise, B. Story, B.F. Boyce, G.R. Mundy and G.D. Roodman. IL-6 enhances hypercalcemia and bone resorption mediated by PTHrP *in vivo*. J. Clin. Invest. 95:2846-2852,1995.

94. Rizzoli, R., J.H.M. Feyen, G. Grau, A.Wohlwend, A.P. Sappino and J-Ph.Bonjour. Regulation of parathyroid hormone-related protein production in a human lung squamous cell carcinoma line. Journal of Endocrinology 143:333-341, 1994.

95. Guise, T.A., S.D.Taylor, T. Yoneda, A.Sasaki, K. Wright, B.F. Boyce, J.M. Chirgwin and G.R. Mundy. PTHrP expression by breast cancer cells enhance osteolytic bone metastases *in vivo*. J. Bone Min. Res. 9(Suppl 1)33: S128a (Abstr.),1994.

96. Kiriyama, T., M.T. Gillespie, J.A. Glatz, S. Fukumoto, J.M. Moseley and T.J. Martin. Transforming growth factor β stimulation of parathyroid hormone-related protein (PTHrP): a paracrine regulator? Mol. and Cell. Endocrinology 92:55-62, 1992.

97. Yoneda, T., P. Williams, C. Dunstan, J. Chavez, M. Niewolna and G.R. Mundy. Growth of metastatic cancer cells in bone is enhanced by bone-derived insulin-like growth factors. J Bone. Min. Res. 10(Suppl 1) P269a: S196a (Abstr.),1995.

FIGURE 1

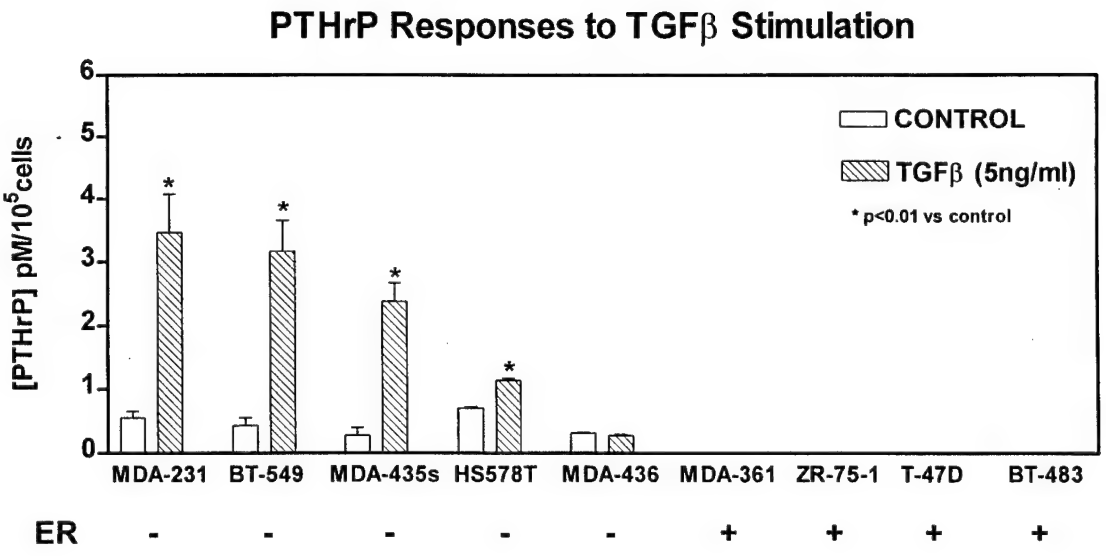


FIGURE 1: PTHrP secretion by 9 different breast cancer cell lines in response to stimulation by TGFβ (5 ng/ml for 48 hours). Cells were plated in 48-well plates at 10^4 cells/ml and grown to confluence. Media was replaced with serum-free DMEM with or without TGFβ (5 ng/ml). Conditioned media were collected after 48 hours. PTHrP concentrations were determined by IRMA and corrected for cell number.All estrogen receptor positive cell lines (ER+), MDA-436, MDA-361, ZR-75-1, T-47D and BT-483, had undetectable concentrations of PTHrP in the conditioned media while all ER- lines, MDA-MB-231, BT-549, MDA-435s, Hs578T, and MDA-436 had low, but detectable PTHrP concentrations in the conditioned media. Of the 5 ER- cell lines that had measurable PTHrP, 4 of these cell lines increased production of PTHrP in response to TGFβ. TGFβ had no effect on PTHrP secretion by ER+ cell lines.

FIGURE 2

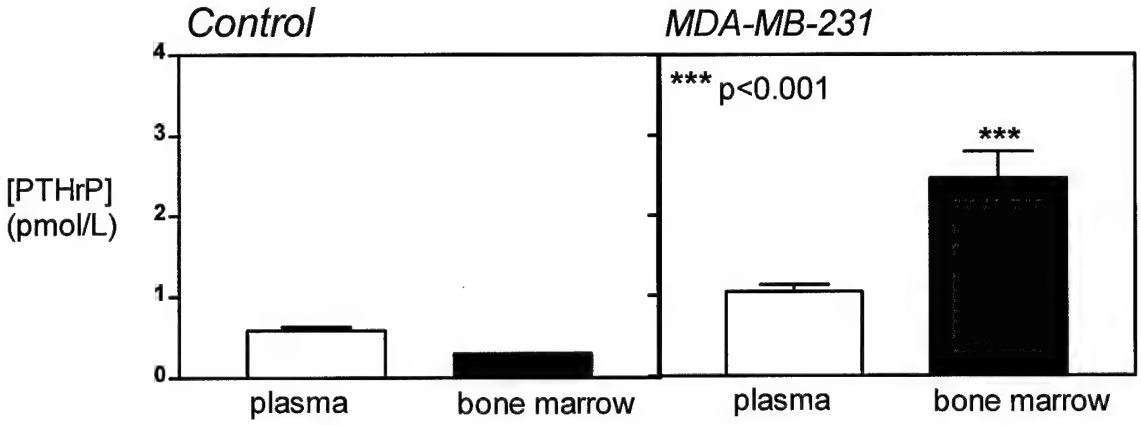


FIGURE 2: Simultaneous PTHrP concentrations in plasma and bone marrow plasma from nontumor-bearing mice (control; left panel) or mice with bone metastases due to MDA-MB-231 tumor (right panel). Bone marrow plasma (indicated as bone marrow in figure) was obtained at the time of sacrifice from femurs with radiologic evidence of bone destruction in mice with MDA-MB-231 tumors or from nontumor-bearing femurs in control mice. Plasma samples were obtained from whole blood collected from respective mice at the same time. N=5 mice/group. Results are expressed as the mean ± the standard error of the mean. ***p<0.001 compared with concomitant plasma sample in mice with MDA-MB-231 tumors.

FIGURE 3

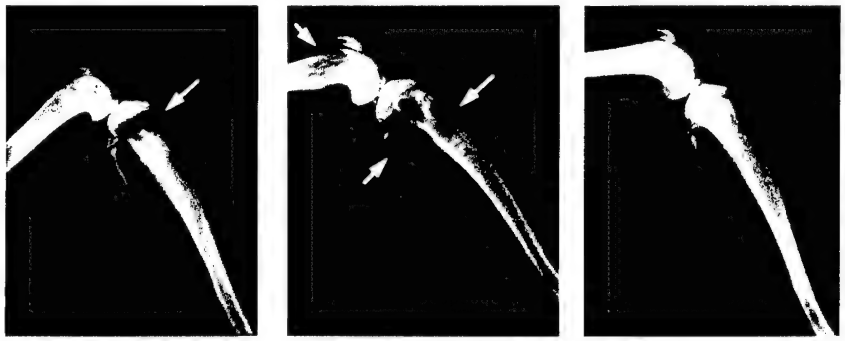


FIGURE 3: Representative radiographs of osteolytic bone lesions in hindlimbs from mice inoculated via the left cardiac ventricle from respective treatment groups. Radiographs were taken 26 days after tumor inoculation with MDA-MB-231 cells. Arrows indicate osteolytic metastases in distal femur, proximal tibia and fibula.

FIGURE 4

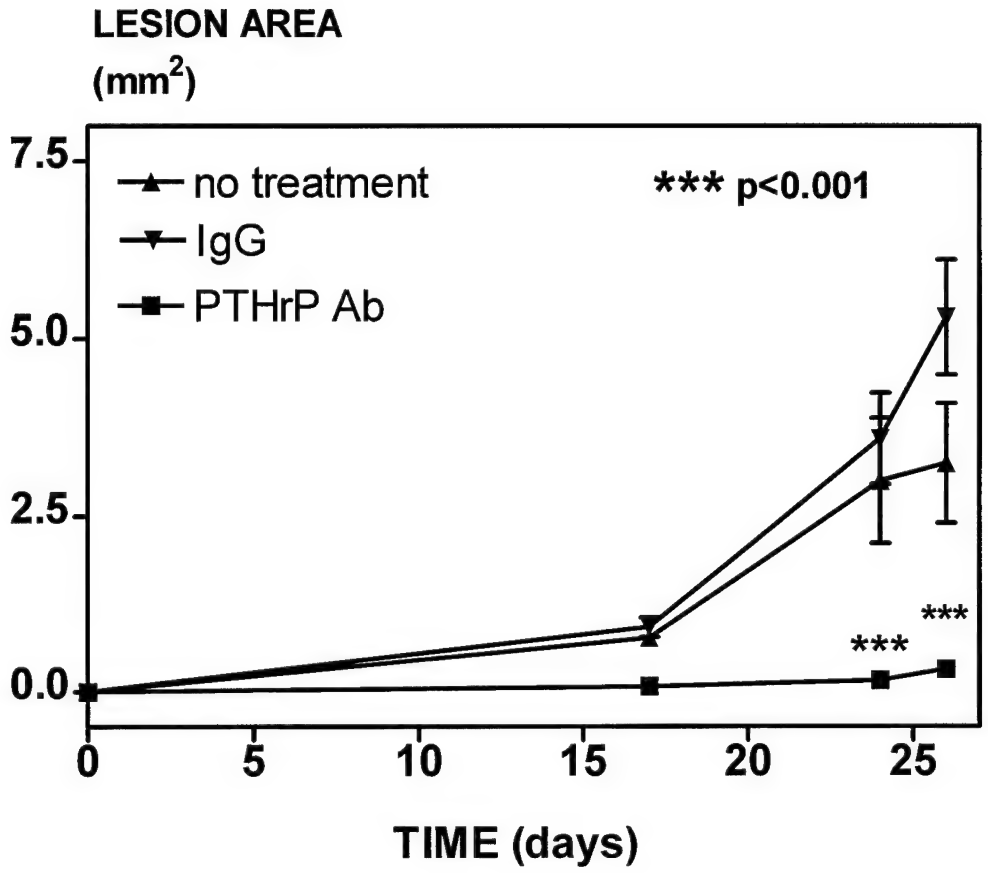


FIGURE 4: Osteolytic lesion area on radiographs as assessed by computerized image analysis. MDA-MB-231 cells were inoculated on day 0, and representative treatment (IgG or PTHrP Ab) started 7 days prior to tumor inoculation and continued twice per week throughout the experiment. Lesion area was measured from long bones of fore- and hindlimbs. N=7 mice/group. All values represent the mean ± SEM. Standard error bars are not visualized at some of the time points as they fall within the size of the symbol.

FIGURE 5

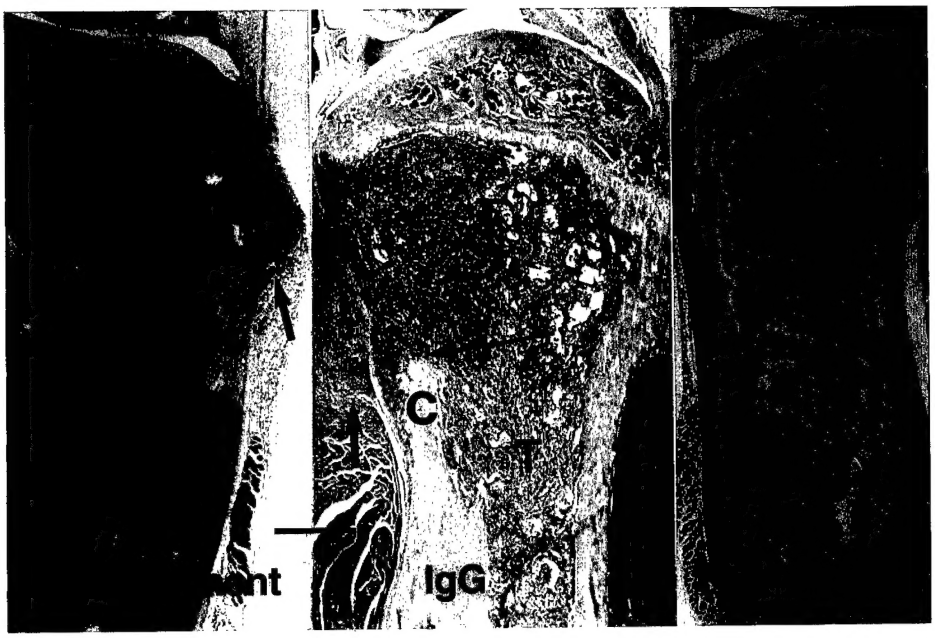


FIGURE 5: Effect of PTHrP antibody on MDA-MB-231 cell metastasis to murine tibiae. MDA-MB-231 breast cancer cells were injected into the left ventricle of nude mice which were sacrificed 26 days later. Mice were also given 75 µg of PTHrP antibody or IgG twice weekly or else received no concomitant treatment. The left hand and middle panels illustrate representative sections of the tibiae from mice given no treatment or IgG. Most of the cancellous bone in the primary and secondary spongiosae has been replaced by metastatic tumor cells (T) which almost completely fill the bone marrow cavity. The cortical bone (C) has also been destroyed by osteoclasts at the proximal ends of the bones (large arrows) in response to the metastatic cancer cells. Tumor cells have spread through the cortical bone into the surrounding soft tissues (small arrows). In contrast, the right hand panel illustrates the tibia from a representative mouse injected with tumor cells and given PTHrP antibody. A relatively small deposit of metastatic tumor (T) is present within the bone marrow cavity distal to the secondary spongiosae. The bone trabeculae at the primary and secondary spongiosae are preserved (small arrows), appear normal and are surrounded by normal bone marrow hematopoietic tissue. The bar represents 555 µm. Hematoxylin, eosin, phloxine and orange G staining.

FIGURE 6

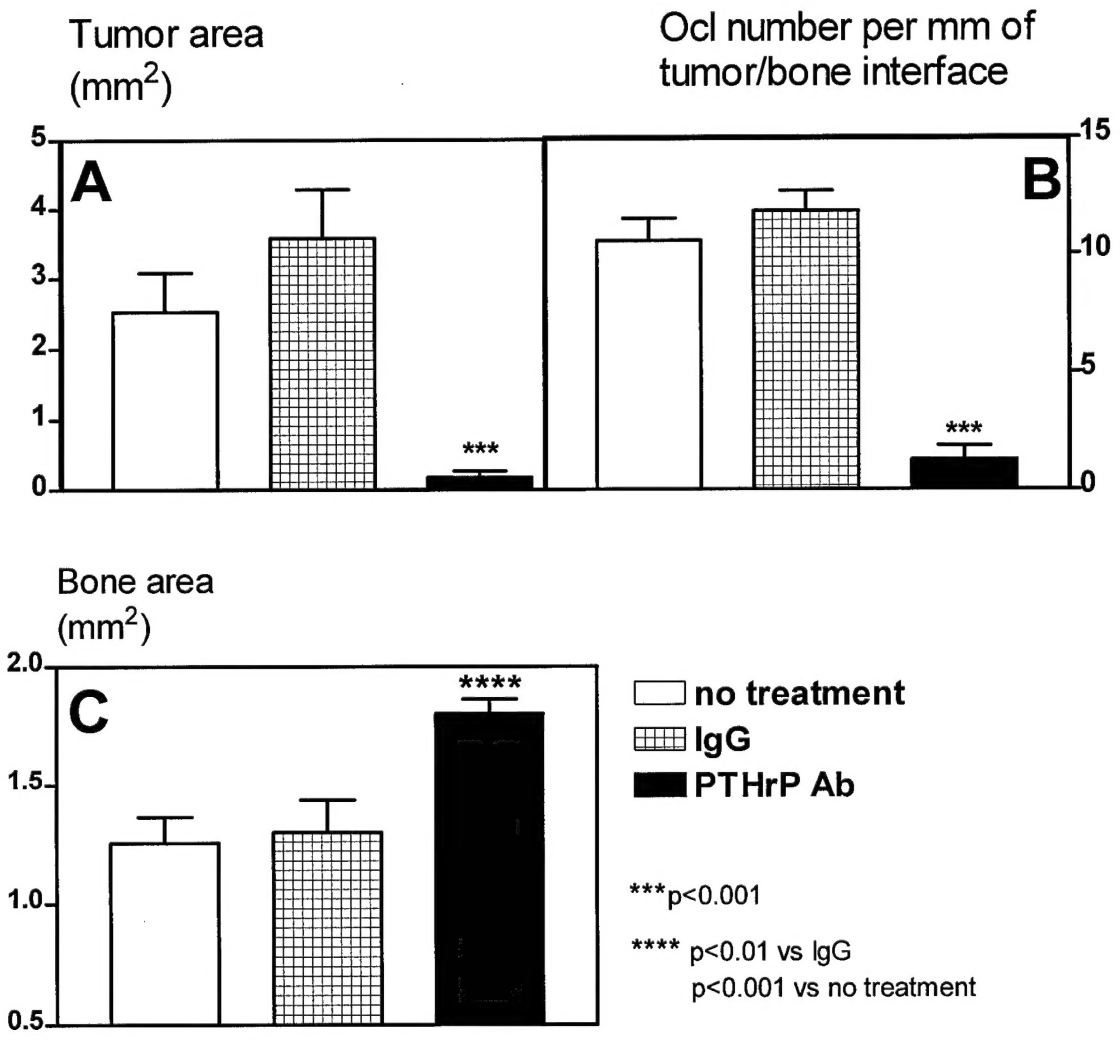


FIGURE 6: Histomorphometric analysis of hindlimbs from tumor-bearing mice with MDA-MB-231 metastatic bone lesions. Data represent measurements from tibiae and femurs of mice from figure 3 that were treated with either PTHrP Ab, IgG or nothing. A) Tumor area (mm^2) from MDA-MB-231 metastatic bone lesions. B) Osteoclast number per mm of tumor adjacent to bone (tumor/bone interface). C) Total bone area (mm^2) was measured in one low-power magnification field (4x) at the head of the tibia or femur, the site of most bone destruction. All values represent the mean ± the standard error of the mean.

FIGURE 7

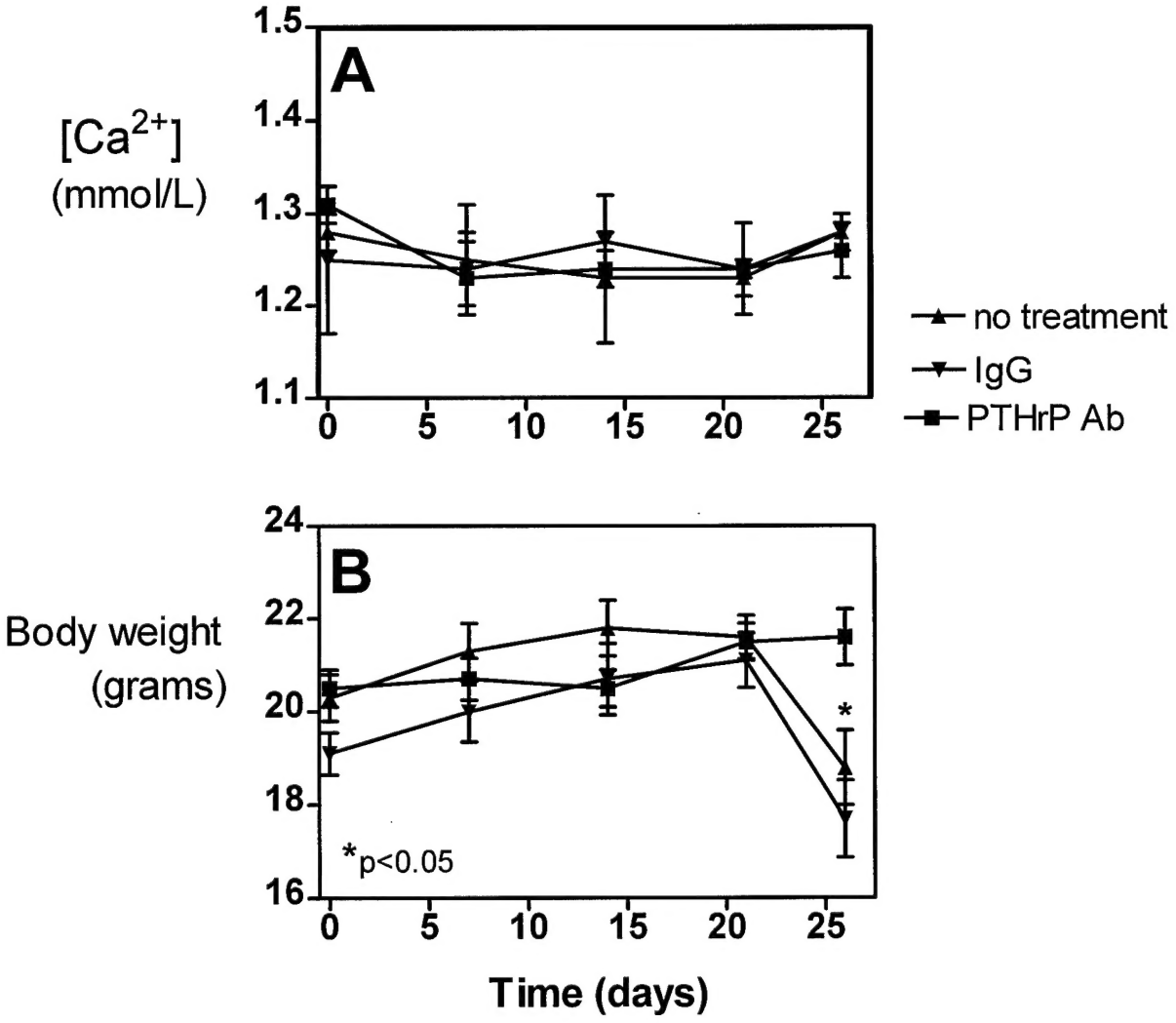
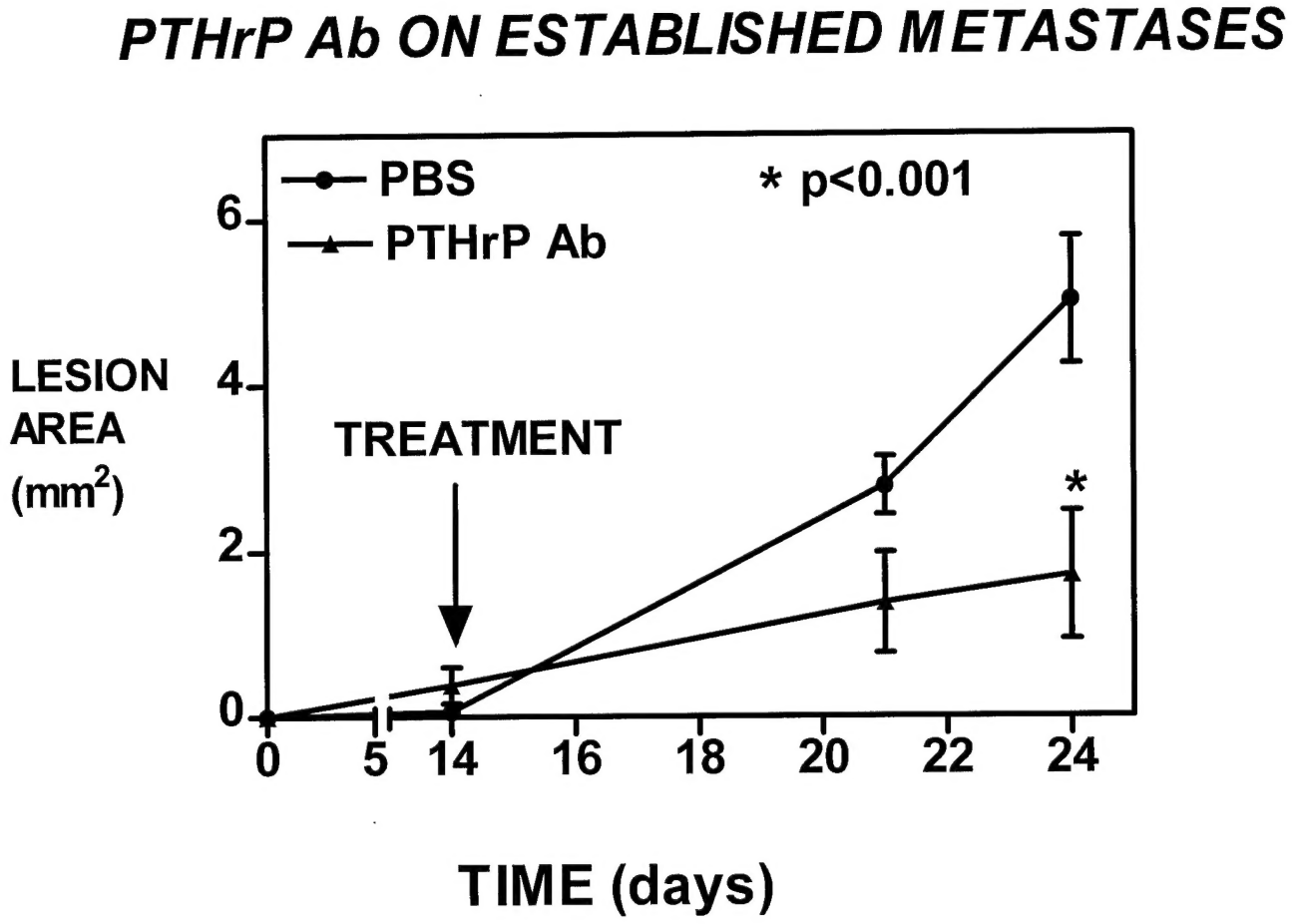


FIGURE 7: Whole blood ionized calcium concentrations [Ca^{2+}] (A) and body weight (B) in tumor-bearing mice from figure 3 that received either PTHrP Ab, control IgG or no treatment. There were no significant differences between the groups with regard to [Ca^{++}] (A) or body weight (B). All values represent the mean ± the standard error of the mean.

FIGURE 8



Area of osteolytic lesions in mice with established bone metastases. Eight female athymic nude mice were inoculated into the left cardiac ventricle on day 0 with nontransfected MDA-MB-231 cells. Osteolytic lesions were evident on radiographs at 2 weeks post inoculation and mice were divided into antibody treatment and control (PBS). Anti-PTHrP-(1-34) antibodies were administered subcutaneously twice per week beginning at this time in doses similar to the previous experiment (70µg IgG).